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Differential expression of three key anthocyanin biosynthetic genes in a color-changing flower, *Viola cornuta* cv. Yesterday, Today and Tomorrow

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Abstract

The natural floral color change in *Viola cornuta* cv. Yesterday, Today and Tomorrow (YTT) provides an opportunity to examine the expression of genes along the anthocyanin biosynthetic pathway in response to known environmental triggers during ontogeny. We have cloned and sequenced gene fragments from three anthocyanin biosynthetic genes, chalcone synthase (*CHS*), dihydroflavonol 4-reductase (*DFR*) and anthocyanidin synthase (*ANS*). Using quantitative reverse-transcriptase-polymerase chain reaction (RT-PCR) we have determined that *CHS* and *DFR* are highly expressed in Stage I white YTT flowers and undergo only moderate increases in expression (25 and 56%, respectively) during the transition to Stage II lavender and Stage III purple flowers. In contrast, *ANS* is expressed at low levels in Stage I flowers and undergoes a dramatic increase (302%) in expression over ontogeny. Interestingly, expression of all three genes is significantly lower in the absence of two environmental triggers, pollination and light, both of which are necessary for floral color change in *V. cornuta*. Our results demonstrate the presence of at least two regulated steps in *V. cornuta* anthocyanin biosynthesis: an early step influenced by pollination and light induces expression of earlier genes (*CHS* and *DFR*) in the anthocyanin biosynthetic pathway, and is necessary but insufficient for floral color change, followed by a second step that affects floral color change by inducing expression of later genes (*ANS*). We have thus identified important environmental cues and developed molecular tools that establish *V. cornuta* as a new model system for the study of the regulation of natural floral color change.

Keywords: Anthocyanin biosynthesis; Floral color change; *Viola*; Light; Pollination

1. Introduction

Anthocyanins are widespread red, blue and purple plant pigments that belong to the most extensively studied group of plant secondary metabolites, the flavonoids. These pigments are ecologically significant, as they provide the visual cues that organisms use to recognize many flowers and fruits for the purposes of pollination and fruit dispersal [1,2]. Anthocyanins are late products of the flavonoid biosynthetic pathway (Fig. 1). Flavonols, which are early products, act as co-pigments and form complexes with anthocyanins to enhance and diversify colors in plant tissues [1].

Anthocyanin production is known to be induced by both developmental and environmental controls. In various taxa, such as *Antirrhinum* [3] and *Petunia* [4], increased steady-state transcription of anthocyanin biosynthetic genes occurs as color is produced in the bud [5]. In many cases, light influences anthocyanin biosynthesis [6]. Light irradiation upon a photoreceptor triggers signal transduction cascades that ultimately lead to the transcriptional activation of anthocyanin biosynthetic genes. Light may also modify floral color by influencing photosynthetic capacity and thus availability of sugars [7], which stabilize anthocyanins in

Abbreviations: *ANS* and ANS, anthocyanidin synthase; *CHS* and CHS, chalcone synthase; *DFR* and DFR, dihydroflavonol 4-reductase; F3H, flavonone 3-hydroxylase; PACC, post-anthesis floral color change; *TUB* and TUB, tubulin; UFGT, UDP-flavonoid glucosyl transferase

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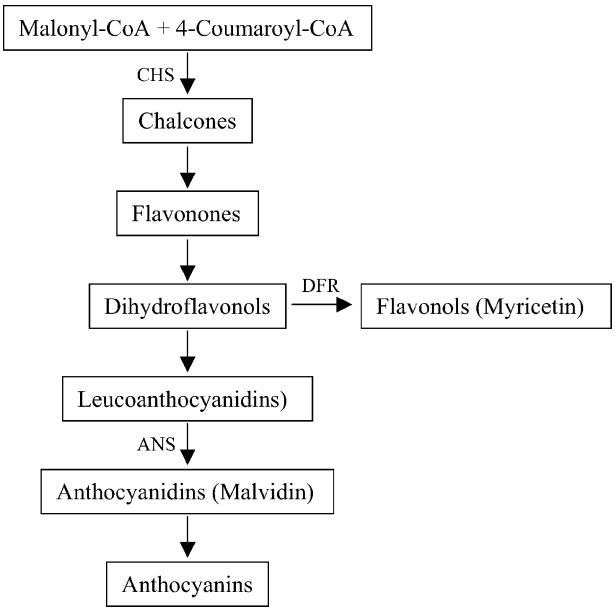

Fig. 1. Simplified schematic of the anthocyanin biosynthetic pathway showing products previously determined to accumulate in *V. cornuta* flowers (myricetin and malvidin) and key enzymes examined in this study (CHS, DFR and ANS).

the vacuole [1]. Pollination can also affect anthocyanin biosynthesis; some flowers, such as *Hibiscus mutabilis* [8] and *Cymbidium* sp. [9], turn red after pollination, presumably due to synthesis of anthocyanins.

In a number of plant taxa such as *Lantana*, *Hibiscus* and *Viola*, fully opened flowers undergo dramatic color changes [10]. These post-anthesis color changes (PACCs) occur in turgid flowers and may affect either a localized portion of the flower or the entire flower. PACC flowers have been richly explored from ecological and evolutionary perspectives [11–14], and light and pollination have been shown to act as environmental triggers for color change. Because the production of pigment occurs in open flowers over a period of several days and can be manipulated by the presence or absence of triggers, PACC flowers provide a useful tool for studies of the regulation of anthocyanin biosynthesis. Indeed, other groups have investigated pigment production in PACC flowers by measuring the activity of the enzyme phenylalanine-ammonia lyase (PAL) [8,9], which catalyzes a reaction that provides the precursor not only for anthocyanin production but also lignin and stilbene synthesis. To date, however, the current links between PACC and pigment biosynthesis are limited to these two studies.

Here we examine the ontogenetic floral color change in *Viola cornuta* cv. Yesterday, Today and Tomorrow (YTT) as a means to pursue the links between environmental cues and anthocyanin gene expression. *Viola* plants grow readily and quickly in the greenhouse [15], and under natural conditions the flowers open white, turning purple within 5–8 days (Fig. 2). The development of color depends on the availability of two important environmental cues, pollination

Fig. 2. Photo of *V. cornuta* cv. Yesterday, Today and Tomorrow (YTT). The three stages of floral color development shown from left to right are Stage III, Stage I, and Stage II.

and light. In the absence of either one of these triggers, the flowers remain white [15]. We have established that YTT color change is based on a gradual accumulation of malvidin (an anthocyanidin) glycosides against a constant background level of myricetin (a flavonol co-pigment) glycosides, and is not due to a change in pH [15] as has been observed in another color-changing flower, *Fuchsia* [16].

These results have directed our investigation toward specific genes involved in anthocyanin biosynthesis. Myricetin is an early product of the anthocyanin biosynthetic pathway, and malvidin is produced at later stages (Fig. 1). Based on these pigment profiles, we have chosen to examine the first step of the anthocyanin biosynthetic pathway, chalcone synthase (*CHS*), and two later steps, dihydroflavonol 4-reductase (*DFR*) and anthocyanidin synthase (*ANS*). The objectives of this report were to (1) identify the cDNA sequences for portions of *CHS*, *DFR*, *ANS* and *tubulin* (*TUB*) from *V. cornuta*, (2) determine the steady-state mRNA levels for *CHS*, *DFR* and *ANS* during *V. cornuta* color change, and (3) measure levels of *CHS*, *DFR* and *ANS* mRNA in response to light and pollination.

2. Materials and methods

2.1. Materials

V. cornuta cv. YTT seeds were obtained from Dr. Jugan Sharma, Waller. Seed Company (Guadalupe, CA). DNase I, TAQ and PCR reagents were obtained from Promega (Madison, WI). Oligonucleotides, reverse transcriptase (RT), TOPO-TA cloning kits and gel electrophoresis reagents were obtained from Invitrogen (Carlsbad, CA). Reagents for RNA isolation and analysis were obtained from Tel-test, Inc. (Friendswood, TX) and Ambion (Austin, TX). Unless indicated, all other chemicals were obtained from Fisher Scientific (Fair Lawn, NJ).

2.2. Flower stages and treatments

V. cornuta flowers at anthesis are white (Stage I). Stage II flowers develop a lavender color that appears 2–5 days after opening [15]. Stage III flowers are completely purple and remain turgid for another 3 days before senescing. Plants were grown as previously described [15].

For the light/dark treatments, five plants that had a significant number of buds likely to open within 3 days were randomly selected. All open flowers were removed at the start of the experiment. Half of each plant was covered with aluminum foil, while the other half was left exposed to light. After 8 days, the aluminum foil was removed and the colors of all flowers judged by petal size and quality to be day three or older (to allow enough time for color to appear) were recorded for plants of both treatments.

For the pollination treatment, age-matched buds on the same plant were arbitrarily selected and labeled to be either un-pollinated or pollinated (*V. cornuta* flowers self-pollinate in bud). A total of 14 pairs of buds on four plants were used in the experiment. Three days before anthesis, undehisced anthers were removed from treatment plants using dissecting forceps. In the control population, anthers were left intact but the gynoecia of the control buds were probed with forceps to control for disturbance in treated plants. Flower color was compared between treatments 5 days after anthesis.

2.3. Total RNA isolation and cDNA synthesis

Flowers were collected for three analyses: ontogeny Stages I, II and III; dark and light; and un-pollinated and pollinated. Each treatment was conducted in duplicate with different sets of tissue from different plants. On average, 40 petals were harvested, the constitutively pigmented nectar guides were excised, and the remaining 200 mg of tissue were frozen immediately in liquid nitrogen. Total RNA was isolated with RNA Stat-60 (Tel-test Inc., Friendswood, TX), and integrity was confirmed by gel electrophoresis in a 6.6 M formaldehyde gel, 1× MOPS buffer (Ambion, Austin, TX). RNA concentration was determined by absorbance at 260 nm, and purity was established with a 260/280 ratio.

To reduce genomic DNA contamination, 2 μg of RNA was treated with 1 U DNase for 30 min at 37 °C in 10 μl of 1× DNase buffer. The reaction was stopped with 1 μl stop solution at 65 °C for 10 min. This RNA was reverse transcribed with 25 U of superscript reverse transcriptase in 20 μl reactions (1× RT buffer, 100 ng random hexamers, 1 mM × 10 mM dNTP) at 42 °C for 50 min followed by 15 min at 72 °C to stop the reaction.

2.4. Gene identification

Polymerase chain reaction (PCR) with 2 μl of the prepared cDNA was carried out in 50 μl in 1× *Taq* reaction buffer containing 2.5 mM $MgCl_2$, 250 mM each dNTP, 800 nM each primer, 4 U *Taq* polymerase (Promega, Madison, WI). The thermocycler program was: 94 °C (5 min); 40 cycles at 94 °C (45 s), 55 °C (45 s), 72 °C (45 s); 72 °C (15 min). Degenerate primer sequences designed from alignments with multiple dicot taxa sequences were used to amplify internal segments of *CHS*, *DFR*, *ANS*, and *TUB* from *V. cornuta* cv. YTT (Table 1). The resulting PCR products were immediately cloned into TOPO-TA vector according to the manufacturer's protocol. Plasmid DNA was isolated from bacteria using Concert Miniprep Kits (Invitrogen, Carlsbad, CA) and restriction digests performed to confirm proper cloning.

Table 1
Degenerate primers derived from related sequences in various species and used to amplify internal segments of *CHS*, *DFR*, *ANS* and *TUB* from *V. cornuta*

(1) *CHS* forward	5′-TAYCARCARGGNTGYTTYGC-3′
(2) *CHS* reverse	5′-GGRTGDGCDATCCARAAVA-3′
(3) *DFR* forward	5′-GCYACNCCYATGGAYTTYGADTC-3′
(4) *DFR* reverse	5′-CRAARTACATCCADSCDGTCATYTT-3′
(5) *ANS* forward	5′-TGGGARGAYTAYTTYTTYCA-3’
(6) *ANS* reverse	5′-RTTRTAYTTNCCRTTRCT-3′
(7) *TUB* forward	5′-AYGAYGCHTTYAAYACHTTYTT-3′
(8) *TUB* reverse	5′-GGRTCRCAYTTDGCCATCAT-3′

Nucleotides are indicated by the IUPAC 1-letter codes (Y = C + T; R = A + G; S = C + G; H = A + T + C; V = A + C + G; D = A + T + G; N = A + C + G + T).

Sequencing reactions were performed with 5.3 μl plasmid DNA from Minipreps, 14 ng M13 reverse primer and 4 μl sequencing mix containing dRhodamine Dye v. 3 (Applied Biosystems, Foster City, CA), for 40 cycles at 96 °C (10 s), 50 °C (5 s), 60 °C (4 min). Reactions were analyzed on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Resulting sequences were aligned using Sequencher Software v.4.0.5 (Gene Codes Corp., Ann Arbor, MI) and gene identity was assessed by similarity searches using Blastx.

All sequences for cDNA fragments have been deposited in Genbank™. Accession numbers are AY294024—*CHS* 390 bp cDNA fragment, AY294025—*DFR* 234 bp cDNA fragment, AY294026—*ANS* 405 bp cDNA fragment, AY294027—*TUB* 509 bp cDNA fragment. The sequence for *CHS* denoted here represents the most frequently detected expressed sequence; additional *CHS* sequences were detected as noted in the text, and the entire set of sequences will be deposited separately (Farzad et al., unpublished data).

2.5. Analysis of gene expression

The same PCR conditions and primer pairs as described above were used for RT-PCR reactions. Quantitative RT-PCR was performed for *CHS*, *DFR*, *ANS* and *TUB* on RNA isolated from three sets of flowers: Stages I, II and III; dark and light; and un-pollinated and pollinated. Initial

estimates of the linear range for RT-PCR for each gene were determined by running reactions over a wide range of PCR cycle numbers (data not shown). Cycle numbers that consistently gave product in the linear range were used for all experiments as follows: *CHS* (28), *DFR* (35), *ANS* (37) and *TUB* (39). Final experiments were performed in duplicate independently—separate petals, separate RT reactions, and duplicate PCR reactions. PCR products were analyzed by gel electrophoresis on 1% agarose gels and visualized after staining with ethidium bromide using AlphaImager Software 2200 v. 5.1 (Alpha Innotech Corp., San Leandro, CA). Expression was quantified by measuring band intensity for each PCR product. Values were standardized to RT-PCR band intensity measurements obtained for tubulin, the constitutive control, from the same RNA samples.

3. Results

3.1. Isolation of partial V. cornuta CHS, DFR, ANS, and TUB cDNA sequences

Because no previous molecular studies have been conducted in *V. cornuta*, we began by identifying gene sequences for key enzymes in the anthocyanin biosynthetic pathway. We isolated and cloned partial *V. cornuta* cDNA fragments of the genes encoding chalcone synthase, dihydroflavonol 4-reductase, and anthocyanidin synthase. We also cloned a partial alpha-tubulin fragment to use as a constitutive control in expression studies. Fig. 3 shows the regions of each gene that were selected for amplification. Degenerate primers were designed to highly conserved sequences based on a consensus derived from alignments of sequences from multiple taxa.

The length of the partial *CHS* clone is 390 bp. We obtained multiple *CHS* sequences suggesting that *V. cornuta CHS* is encoded by a multi-gene family (Farzad et al., manuscript in preparation), as has been observed in a number of taxa. The dominant sequence obtained by RT-PCR from petal tissue was used for analysis here. The amplified region includes the conserved CHS catalytic site for the enzyme reported for other taxa [17], which facilitated positive identification of our sequence through BLAST analysis. *V. cornuta* CHS identity was confirmed by the high homology to other CHS sequences from *Petunia hybrida*, *Zea mays*, *Antirrhinum majus*, *Perilla frutescens*, *Vitis vinfera*, *Ipomoea purpurea*, and *Arabidopsis thaliana* (Table 2 and sequence alignments as shown in Fig. 4A). Two (Cys^{164} and Phe^{215}) of the four strictly conserved residues (also His^{303} and Asn^{336}) that are important for catalytic activity of CHS and CHS-related enzymes [17] fall within the region of *CHS* that we have amplified, and their presence further supports the gene identification.

DFR is a member of the short chain dehydrogenase/reductase (SDR) superfamily [18]. Substrate specificity is determined by a very small region of *DFR* [19], and the 234 bp cDNA fragment that we amplified from *V. cornuta DFR* region spans these important residues. BLAST searches consistently returned *DFR* sequences from other taxa with high levels of identity and similarity (Table 2; Fig. 4B). Analysis of the *V. cornuta* sequence demonstrates that *V. cornuta* DFR, like *Petunia* DFR, has a key glutamic acid residue instead of the more common asparagine within

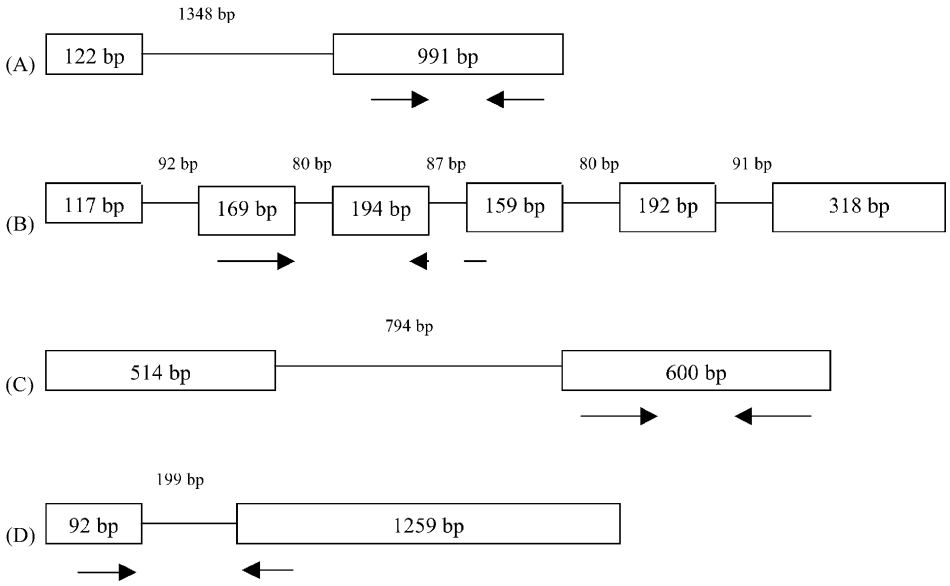

Fig. 3. Schematic representation of the regions of each gene used to design degenerate primers. White boxes and lines depict exon and intron sizes, respectively. The arrows represent upstream and downstream primer locations used for PCR. The broken arrow in B indicates a primer that spans the exon boundaries, excluding the third intron. (A) *P. hybrida CHSA* (accession no. X14591). (B) *A. thaliana DFR* (accession no. AB033294). (C) *Ipomoea nil ANS* (accession no. AB073924). (D) *A. thaliana alpha-6-tubulin* (accession no. M84699).

Table 2
Percent similarity and identity of *V. cornuta CHS*, *DFR* and *ANS* amino acid sequences with related sequences from other species

Gene	Species	GenBank accession no.	Amino acid (% identity)	Amino acid (% similarity)
CHS	*V. cornuta* cv. YTT	(AY294024)	100	100
	P. hybrida	(P08894)	79	85
	Z. mays	(P24825)	79	85
	A. majus	(CAA27338)	79	85
	P. frutescens	(O04111)	81	86
	V. vinifera	(AAB72091)	83	86
	I. purpurea	(AAB41103)	79	86
	A. thaliana	(AAM65314)	79	86
DFR	*V. cornuta* cv. YTT	(AY294025)	100	100
	P. hybrida	(P14720)	71	88
	Z. mays	(P51108)	74	89
	A. majus	(P14721)	58	88
	P. frutescens	(BAA19658)	71	92
	V. vinifera	(CAA53578)	78	91
	I. purpurea	(BAA36407)	70	89
	A. thalian	(BAA85261)	81	91
ANS	*V. cornuta* cv. YTT	(AY294026)	100	100
	P. hybrida	(S36233)	77	84
	Z. mays	(P41213)	50	68
	A. majus	(N/A)		
	P. frutescens	(BAA20143)	75	82
	V. vinifera	(P51093)	77	82
	I. purpurea	(AAB84049)	75	83
	A. thaliana	(AAA32892)	76	83

The *candi* locus encoding ANS in *Antirrhinum* has been identified but there is no sequence available in GenBank.

its active site; this in turn suggests that *V. cornuta* DFR, like *Petunia* DFR, cannot utilize DHK [19].

ANS is a member of the very large dioxygenase family. The *V. cornuta ANS* cDNA fragment is 405 bp. BLAST analysis preferentially returned *ANS* sequences from other taxa with high levels of identity and similarity (Table 2; Fig. 4C). Additionally, *V. cornuta* ANS retains the conserved His (H) and Asp (D) residues that are important for ferrous ion binding within our amplified region [20,21]. Further confirmation of the identity of our sequence as an anthocyanidin synthase rather than another member of the larger dioxygenase family comes from tertiary modeling predictions from SWISS-MODEL [22,23]; structural alignments returned only the three ANS structures within the Molecular Modeling Database (data not shown).

The length of the *V. cornuta TUB* cDNA fragment is 783 bp and the derived protein sequence of the fragment is consistently 97–99% identical and similar to alpha-tubulin genes from other flowering plant taxa (data not shown). In *V. cornuta*, our cloned regions of *CHS*, *DFR*, *ANS* and *TUB* permit us to undertake comparative analysis of expression patterns of key anthocyanin biosynthetic enzymes across different stages and under different environmental treatments.

3.2. Expression over ontogeny

We examined expression levels of *CHS*, *DFR* and *ANS* during three stages of floral color development in *V. cornuta* flowers using RT-PCR. Relative expression levels were compared to *TUB* to permit quantitative comparison for each enzyme across the three stages. Given our observation that multiple *CHS* genes exist in *V. cornuta* and our interest in total *CHS*, *DFR*, *ANS* and *TUB* expression, we used degenerate primers for the RT-PCR experiments to permit detection of expression of multiple gene copies and alleles. The absence of any product, including longer intron-spanning segments in *DFR* and *ANS*, in reactions lacking reverse transcriptase confirms the absence of genomic DNA contamination in our cDNA samples (Fig. 5 and data not shown). Our data demonstrate that *CHS* and *DFR* are expressed throughout all three stages of development (Fig. 5). Data from two representative independent experiments (separate sets of petal tissue and separate RT reactions were followed by PCR reactions in duplicate) were used to calculate changes in expression. *CHS* expression increases only slightly as color develops intensity (25% from Stages I to III), while *DFR* expression shows a more significant increase as color develops (56% from Stages I to III) (Table 3). *ANS*, on the other hand, is expressed at a low level in Stage I flowers, and expression increases dramatically as purple coloration appears in Stage III flowers (302%) (Fig. 5 and Table 3). Having determined that *ANS* expression is strongly regulated over ontogeny led us to question the role of environmental cues in triggering expression of genes along the anthocyanin biosynthetic pathway.

3.3. Expression in light and dark treatments

Stage I flowers (white) phenotypically resemble flowers grown in the absence of light and pollination, while flowers exposed to these environmental triggers turn purple [15]. We tested the effect of light on gene expression by examining the expression patterns of *CHS*, *DFR* and *ANS* in flowers kept in the dark or exposed to light for an equal number of days. *CHS*, *DFR*, and *ANS* are all expressed in light treatments (Fig. 6), corresponding to the ontogeny data for Stage III flowers (Fig. 5). Same-aged flowers kept in the dark do not develop pigment, and expression of all three genes is

Table 3
Quantitation of RT-PCR results showing relative percent increases in gene expression from Stage I–II to Stage III

	Percent change from Stage I to II	Percent change from Stage I to III
CHS	29% (5)	25% (15)
DFR	16.5% (6.6)	56.5% (6.6)
ANS	203% (5)	302% (25.2)

Data are shown as the arithmetic mean of percent increase (standard error) from two independent experiments. All numbers were standardized to *TUB* expression levels in each stage.

considerably less than that observed in light-treated flowers (Fig. 6), as has been previously observed in *Arabidopsis* [24]. Average levels of expression in dark-treated flowers are 88.5% (*CHS*), 76% (*DFR*) and 91.5% (*ANS*) lower than in light-treated flowers (Table 4). It is notable that the low levels of *CHS* and *DFR* expression in dark-treated (white) flowers differ from the more abundant expression observed in Stage I (white) flowers (Fig. 5).

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                                  *
    1 YTT              YQQGCFAGGTVLRLA KDLAENNRGARVLVV CSEITAVTFRGPSDS
    2 Maize            YQQGCFAGGTVLRVA KDLAENNRGARVLVV CSEITAVTFRGPSES
    3 Antirrhinum      YQQGCFAGGTVLRMA KDLAENNAGARVLVV CSEITAVTFRGPADT
    4 Perilla          YQQGCFAGGTVLRMA KDLAENNAGARVLVV CSEITAVTFRGPSES
    5 Petunia          YQQGCFAGGTVLRLA KDLAENNKGARVLVV CSEITAVTFRGPNDT
    6 Arabidopsis      YQQGCFAGGTVLRIA KDLAENNRGARVLVV CSEITAVTFRGPSDT
    7 Vitis            YQQGCFAGGTVLRLA KDLAENNAGSRVLVV CSEITAVTFRGPSDT
    8 Ipomoea          YQQGCFAGGTVIRLA KDLAENNKGARVLVV CSEITAVTFRGPSDA
                                    *
    1 YTT              HLDSMVGQALFGDGA AAVIVGADA-DLTVE RPLFHIVSAAQTILP
    2 Maize            HLDSLVGQALFGDGA AAVVVGADP-DDRVE RPLFQLVSAAQTILP
    3 Antirrhinum      HLDSLVGQALFGDGA AAVIVGSDP-VVGVE RPLFQIVTAAQTLLP
    4 Perilla          HLDSLVGQALFGDGA AAVIVGSDP-VVGVE RPLFQLVSAAQTILP
    5 Petunia          HLDSLVGQALFGDGA GAIIIGSDP-IPGVE RPLFELVSAAQTLLP
    6 Arabidopsis      HLDSLVGQALFSDGA AALIVGSDPDTSVGE KPIFEMVSAAQTILP
    7 Vitis            HLDSLVGQALFGDGA AAVIIGADP-DTKIE LPLFELVSAAQTILP
    8 Ipomoea          HLDSLVGQALFGDGA AALIIGSDP-DPDLE RPLFQLVSAAQTILP

    1 YTT              DSEGAIDGHLREVGL TFHLLKDVPGLISKN IEKSLVEAFNP
    2 Maize            DSEGAIDGHLREVGL TFHLLKDVPGLISKN IGRALDDAFKP
    3 Antirrhinum      DSHGAIDGHLREVGL TFHLLKDVPGLISKN IEKSLKEAFDP
    4 Perilla          DSDGAIDGHLREVGL TFHLLKDVPGLISKN IEKSLKEAFGP
    5 Petunia          DSHGAIDGHLREVGL TFHLLKDVPGLISKN IEKSLEEAFKP
    6 Arabidopsis      DSDGAIDGHLREVGL TFHLLKDVPGLISKN IVKSLDEAFKP
    7 Vitis            DSEGAIDGHLREVGL TFHLLKDVPGLISKN IEKSLVEAFTP
(A) 8 Ipomoea          DSGGAIDGHLREVGL TFHLLKDVPGLISKH IEKSLNEAFQP

    1 YTT              PMDFESKDPENEVIK PTVNGVLDIMRACAK AKTVRRVVFTSSAGT
    2 Maize            PMDFLSKDPENEVIK PTVEGMISIMRACKE AGTVRRIVFTSSAGT
    3 Antirrhinum      SMEFDSVDPENEVIK PTIDGMLNIIKSCVQ AKTVKKFIFTTSGGT
    4 Perilla          PMDFESDDPENEVIK PTVDGMLSIMRSCTK AKTVKKLIFTNSAGT
    5 Petunia          PMDFESKDPENEVIK PTVRGMLSIIESCAK ANTVKRLVFTSSAGT
    6 Arabidopsis      PMDFESKDPENEVIK PTVNGMLGIMKACVK AKTVRRFVFTSSAGT
    7 Vitis            PMDFESKDPENEVIK PTIEGMLGIMKSCAA AKTVRRLVFTSSAGT
    8 Ipomoea          PVDFVSDDPQNEIIR PAVKGILSIINSCAK AKTVKRLVFTSSAVT

                       *
    1 YTT              VDVEEPKKPVYDESN WSDLEFVRAVKMTAW MYF
    2 Maize            VNLEERQRPVYDEES WTDVDFCRRVKMTGW MYF
    3 Antirrhinum      VNVEEHQKPVYDETD SSDMDFINSKKMTGW MYF
    4 Perilla          LNVEEHQKPVYNEAN WSDLDFIYSKKMTGW MYF
    5 Petunia          LDVQEQQKLFYDQTS WSDLDFIYAKKMTGW MYF
    6 Arabidopsis      VNVEEHQKNVYDEND WSDLEFIMSKKMTGW MYF
    7 Vitis            VNIQEHQLPVYDESC WSDMEFCRAKKMTAW MYF
(B) 8 Ipomoea          LIVQGNPKPVYDESS WSDLDLIYAKKMPGW MYF
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Fig. 4. Comparison of deduced amino acid sequences of cloned *V. cornuta CHS*, *DFR*, and *ANS* gene fragments with related sequences from other species. (A) Alignment of amplified *CHS* fragment from *V. cornuta* with equivalent portions of *CHS* from *Z. mays*, *A. majus*, *P. frutescens*, *P. hybrida*, *A. thaliana*, *V. vinfera* and *I. purpurea*. The sequences are the same as those presented in Table 2. The asterisks above Cys (C) and Phe (F) represent absolute conservation important for catalytic activity. The bold residues assist active site folding. The bold/italic residues specify CHS rather than stilbene synthase cyclization. (B) Alignment of amplified fragment of *DFR* from *V. cornuta* with equivalent portions of *DFR* from designated taxa. The Ser (S) and Tyr (Y) in bold are completely conserved in the DFR superfamily. The asterisk denotes the Asp (D) residue mentioned in the discussion. (C) Alignment of amplified fragment of *ANS* from *V. cornuta* with equivalent portions of *ANS* from designated taxa. The conserved His (H) and Asp (D) residues are in bold.

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1 YTT            PEDKRDLSIWPTKPS NYTEVTSEYARQLRG LATKIFSALSVGLG-
2 Maize          PDGLADHALWPAYPP DYIAATRDFGRRTRD LASTLLAILSMGLLG
3 Perilla        PEHKTDLSIWPTKPP DYIPATSEYAKQLRA LATKILSVLSIGLG-
4 Petunia        PEDKRDLSIWPKNPT DYTPATSEYAKQIRA LATKILTVLSIGLG-
5 Arabidopsis    PSSIRNPSKWPSQPP KIRELIEKYGEEVRK LCERLTETLSESLG-
6 Vitis          PEDKRDMTIWPKTPS DYVPATCEYSVKLRS LATKILSVLSLGLG-
7 Ipomoea        PEDKTDLSIWPKNAS DYIDATREYAKQLRA LATKVLAVLSLGLG-

1 YTT            -----------LEES RLEKEVGGMEDLLLQ MKINYYPKCPQPWLA
2 Maize          TDRGDRGDALEKALT TTTTRTAADDDLLLQ LKINYYPRCPQPELA
3 Perilla        -----------LEKG RLEKEVGGAEDLIVQ MKINFYPKCPQPELA
4 Petunia        -----------LEEG RLEKEVGGMEDLLLQ MKINYYPKCPQPELA
5 Arabidopsis    -----------LKPN KLMQALGGGDKVGAS LRTNFYPKCPQPQLT
6 Vitis          -----------LEEG RLEKEVGGMEELLLQ KKINYYPKCPQPELA
7 Ipomoea        -----------LEEG RLEKEVGGMEELLLQ MKINYYPKCPQPELA

1 YTT            LGVEAHTDISALTFI LHN-MVPGLQLFXXG KWVTAKCVPNSIIMH
2 Maize          VGVEAHTDVSALSFI LHN-GVPGLQVLHGA RWVTARHEPGTIIVH
3 Perilla        LGWEAHTDVSALTFI LHN-MVPGLQLFYED KWVTAKCVPNSIIMH
4 Petunia        LGVEAHTDVSALTFI LHN-MVPGLQLFYEG QWVTAKCVPNSIIMH
5 Arabidopsis    LGLSSHSDPGGITIL LPDEKVAGLQVRRGD GWVTIKSVPNALIVN
6 Vitis          LGVEAHTDVSALTFI LHN-MVPGLQLFYEG KWVTAKCVPNSIIMH
7 Ipomoea        LGVEAHTDVSALTFI LHN-MVPGLQLFYGG KWVTAKCVPNSIIMH

1 YTT            IGDTIEILSNGKY
2 Maize          VGDALEILSNGRY
3 Perilla        IGDTLEILSNGKY
4 Petunia        IGDTIEILSNGKY
5 Arabidopsis    IGDQLQILSNGIY
6 Vitis          IGDTIEILSNGKY
(C) 7 Ipomoea    VGDTVEILSNGKY
```

Fig. 4. (*Continued*).

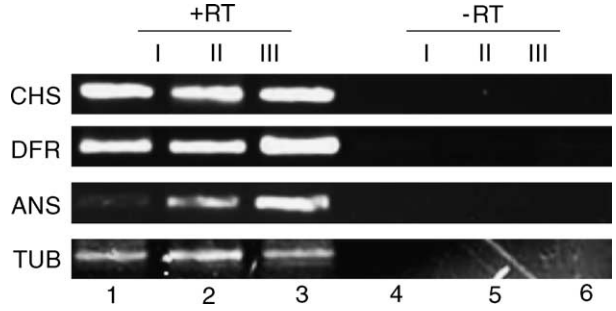

Fig. 5. RT-PCR results from a representative experiment showing expression of *CHS*, *DFR*, *ANS* and *TUB* across *V. cornuta* Stages I, II and III. RT: control lacking reverse transcriptase.

Table 4
Quantitation of RT-PCR results showing relative percent change in gene expression between dark and light flowers

	Percent difference between dark and light treatments
CHS	88.5% (3.5)
DFR	76% (6.1)
ANS	91.5% (8.55)

Data are shown as the arithmetic mean of percent increase (standard error) from two independent experiments. All numbers were standardized to *TUB* expression levels in each stage.

3.4. Expression in un-pollinated and pollinated treatments

We tested the influence of pollination on *CHS*, *DFR*, and *ANS* gene expression by comparing nonpollinated to pollinated flowers grown in the light for an equal number of days. The nonpollinated flowers, like the dark-treated flowers, remain white, and *CHS*, *DFR*, and *ANS* are all expressed at

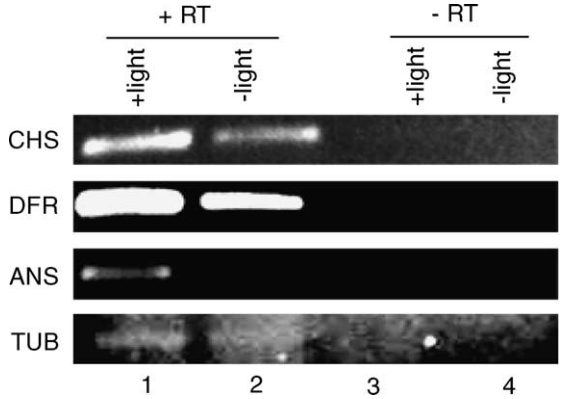

Fig. 6. RT-PCR results showing expression of *CHS*, *DFR*, *ANS* and *TUB* in dark and light-treated plants.

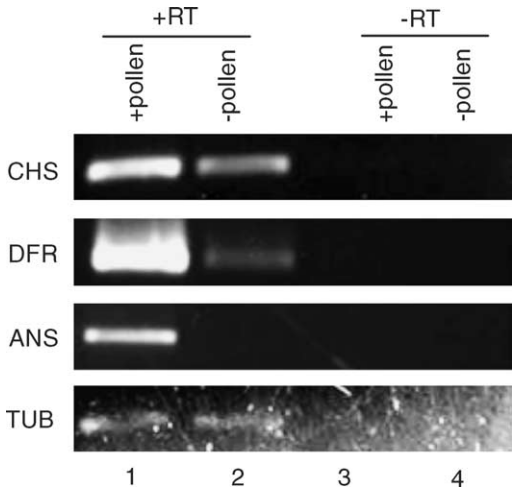

Fig. 7. RT-PCR results showing expression of *CHS*, *DFR*, *ANS* and *TUB* in plants prevented from pollination and allowed to self-pollinate.

Table 5
Quantitation of RT-PCR results showing relative percent changes in gene expression between unpollinated and pollinated flowers

	Percent difference between unpollinated and pollinated treatments
CHS	71.5% (20.7)
DFR	59% (8.5)
ANS	75% (25.2)

Data are shown as the arithmetic mean of percent increase (standard error) from two independent experiments. All numbers were standardized to *TUB* expression levels in each stage.

lower levels than in flowers that turn purple (Fig. 7). Average levels of expression in nonpollinated flowers are 71.5% (*CHS*), 59% (*DFR*) and 75% (*ANS*) lower than in pollinated flowers (Table 5). Thus, the pollination results differ from the expression patterns observed over ontogeny and are similar to those obtained from the dark/light treatments.

4. Conclusion

4.1. *V. cornuta contains homologs of CHS, DFR, and ANS*

We have established the patterns of expression for three anthocyanin biosynthetic genes over color change in PACC flowers in response to environmental triggers during ontogeny. *V. cornuta* is ideal for studies linking environmental cues and regulation of anthocyanin biosynthesis because different colors appear predictably within the same flower and we can manipulate the development of color by removing light and pollination cues [15].

Partial gene sequences for *V. cornuta CHS*, *DFR*, *ANS* and *TUB* were cloned from cDNA using degenerate primers designed against related sequences from other plant taxa. CHS, DFR and ANS are all members of larger enzyme superfamilies. BLAST searches and alignments with *V. cornuta* sequences against numerous other dicot sequences confirmed that we had obtained the specific gene sequence for each gene, and not that of a related superfamily member. This observation was further supported for ANS by protein modeling of the ANS fragment identified.

4.2. *Identification of ANS as a key regulated late stage enzyme in anthocyanin biosynthesis*

We have determined that *ANS* is a key regulated step in *V. cornuta* floral color change during development. The minimal amount of expression in Stage I flowers increases dramatically over ontogeny, reaching a maximum in Stage III flowers (Fig. 5) [15]. *CHS* and *DFR*, on the other hand, are expressed throughout development (Fig. 5). We can conclude that both genes are activated in bud prior to anthesis, because they are present in Stage I flowers. Yet *DFR* gene expression increases to a greater extent over ontogeny than does *CHS* gene expression, suggesting that *DFR* is also up-regulated during floral color development. Clearly in *V. cornuta*, as in other dicots, there is a distinction between regulation of general flavonoid genes (*CHS*) and those specific to anthocyanin biosynthesis (*DFR* and *ANS*). What differs across various dicot taxa is the starting point for late regulation. In *Petunia*, *Antirrhinum* [25], and *Vitis* [26], regulation of anthocyanin biosynthesis begins at *DFR*, *F3H* and *UFGT*, respectively. In *Viola*, however, our results indicate that while early regulatory events are activated to produce CHS and DFR in bud, a second regulatory event activates production of ANS as the key enzyme to produce color in open flowers. Only in a few plants, such as *Sarracenia* [27] and *Perilla* [28] is *ANS* expression reported to be the key regulated step.

4.3. *Evidence for light control of two regulatory checkpoints in anthocyanin biosynthesis*

A role for these two regulatory checkpoints in anthocyanin biosynthesis in *V. cornuta* is strongly supported by several independent lines of evidence. First, we know that light and pollination signals are first received in buds. Preliminary experiments to arrive at a suitable protocol for anther removal demonstrated that anthers must be removed in bud to prevent self-pollination and color development (data not shown). These observations are corroborated by our molecular analyses demonstrating that CHS and DFR expression increase in response to light and pollination cues in Stage I flowers (Figs. 5–7). Our light/dark data are similar to those obtained in analyses of *Vitis* [26] and *Perilla* [29], in which all anthocyanin biosynthetic genes examined show considerably lower expression in the dark. In most plants, such as *Petunia* [4], *Petroselinum* [30], and *Sinapsis* [31], light regulates at least one the anthocyanin biosynthetic genes. Thus, although Stage I flowers are still unpigmented and white, the first necessary triggers have been received in bud to

initiate gene expression along the anthocyanin biosynthetic pathway.

In addition, we know that these early triggers are insufficient to produce color development. The action of a second regulatory event is indicated by the moderate increase in *DFR* expression and the dramatic increase in *ANS* expression later in ontogeny corresponding to the development of purple color. Preliminary data suggests that this second post-anthesis trigger may also be light. When late bud and early Stage I flowers (Day 1—the day of anthesis) are wrapped in foil to prevent further light exposure, a small minority of the flowers (2 of 10 flowers) developed color comparable to that of age-matched control flowers exposed to light. However, when the same treatment is applied to late Stage I or early Stage II flowers (Days 2–3), most (11 of 13 flowers) developed color comparable to controls (M. Farzad, unpublished data). Additionally, open *V. cornuta* flowers covered with a fine mesh to exclude insect pollinators developed a white and purple-checkered pattern: the regions where light was accessible turned purple, while those hidden by mesh remained white [15]. These data suggest that exposure to light through Stage I is required to fully activate anthocyanin biosynthesis. The exact timing of tissue receptivity to light for *ANS* induction has not been established, although our current data provide evidence that exposure of flowers to light approximately 18–30 h after anthesis is sufficient to induce expression of *ANS*, similar to the time frame of 16–24 h after irradiation observed for the activation of flavonoid biosynthetic genes in *Zea* [32], *Petroselinum* [33], and *Arabidopsis* [24]. The delay between exposure to a signal and the appearance of pigment suggests that the light signal in all of these flowers is ultimately interpreted by a series of molecular steps.

Information about the influence of pollination on floral color development is currently restricted to measurements of hormones associated with pollination, such as ethylene and gibberellic acid (GA_3) [34–36]. Our previous research has shown that blocking ethylene synthesis in *V. cornuta* did not prevent the development of floral color [15]. Thus, while pollination is a key trigger to floral color change [13], further research into the molecular control of anthocyanin biosynthesis by this signal will clearly be fruitful.

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